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(54) Title: SYNTHESIS OF STEREOSPECIFIC OLIGONUCLEOTIDE PHOSPHOROTHIOATES

(57) Abstract

Disclosed is a method for synthesizing of stereospecific (Rp) phosphorothioate oligonucleotides. In this method, a primer comprising a plurality of deoxyribonucleotides and a ribonucleotide at the 5' terminal or 5' penultimate position, is annealed to a template. The structure is contacted with a mixture of deoxynucleoside α-triphosphate Sp diastereomers and a DNA polymerase to form a PS-Rp oligodeoxynucleotide extension which is liberated as a single-stranded PS-Rp oligonucleotide by cleavage after the ribonucleotide in the primer. Also disclosed are PS-Rp oligonucleotides and oligonucleotides prepared according to this method.

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SYNTHESIS OF STEREOSPECIFIC OLIGONUCLEOTIDE PHOSPHOROTHIOATES

BACKGROUND OF THE INVENTION

This invention relates to antisense oligonucleotides. More specifically, this invention relates to methods of preparing stereospecific phosphorothioate oligonucleotides.

Phosphorothioate analogs of oligodeoxynucleotides (PS oligonucleotides) are known to be useful as antisense tools (see, e.g., Agrawal et al. (1989) *Proc. Natl. Acad. Sci. (USA)* 86:7790-7794). These analogs have at least one non-bridging oxygen atom that has been substituted for a sulfur atom on the phosphate group in each internucleotide phosphate linkage. Such a modification is a conservative substitution which increases nuclease resistance without significantly impairing the hybridization of the antisense molecule with target mRNA.

PS oligonucleotides have been chemically synthesized using either the phosphoramidite or the H-phosphonate approach (both reviewed in *Methods in Molecular Biology* (Agrawal, ed.) Volume 20, Humana Press, (1993) Totowa, NJ). Such preparative methods introduce a chiral center at each internucleotide linkage that can lead to the formation of 2^n diastereomers per n internucleotide linkages, and generally result in a mixture of about 40% Rp and about 60% Sp diastereoisomers.

Although phosphorothioates have many useful characteristics, such as the ability to initiate RNase H activity upon hybridization and to inhibit reverse transcriptase, and increased nuclease resistance, the presence of both Rp and Sp diastereomers in a phosphorothioate product may be problematic for several reasons. Rp and Sp diastereosomers may have different biophysical properties, such as different affinities for single-stranded and double-stranded nucleic acids (see, e.g., Cossick et al. (1985) Biochem. **24**:3630-3638; Kim et al. (1992) FEBS Lett. **314**:29-32). Hence, it is desirable under certain circumstances to prepare only one diastereomer. In addition, the presence of both diastereomers in the mixture makes difficult the resolution of PS-oligonucleotides from each other and from other unwanted reaction products by polyacrylamide gel electrophoresis and HPLC, as peaks resulting from these chromatographic methods tend to be broader than their phosphodiester-linked counterparts. Furthermore, the lack of stereospecificity is, for steric reasons, partially responsible for the lower melting temperature (T_m) of PS DNA/RNA duplexes relative to phosphodiester linked DNA-RNA duplexes (LaPlanche et al. (1986) *Nucleic Acids Res.* **14**:9081). Accordingly, in the context of new drug development, preparation of such analogs may require that the issue of stereochemical purity be successfully addressed.

Thus, what is needed are methods of synthesizing stereospecific (Rp or Sp)

diastereomers of phosphorothioate oligonucleotides.

SUMMARY OF THE INVENTION

This invention provides, in one aspect, a method of synthesizing the stereospecific Rp diastereomer or isomer of a phosphorothioate oligonucleotide. As used herein, the term "phosphorothioate oligonucleotide" or "PS oligonucleotide" is intended to encompass at least five nucleotides covalently linked 3' to 5' via phosphorothioate internucleotide linkages, and including both Sp and Rp isomers. "PS-Rp oligonucleotide" refer to only the stereospecific (Rp) isomer of a PS oligonucleotide.

To produce a PS-Rp oligonucleotide according to the method of the invention, a nucleotidic primer is annealed to a complementary oligodeoxynucleotide template having 5' and 3' termini, thereby forming a partially double-stranded/partially single-stranded structure. The primer also has 3' and 5' termini and includes a nucleic acid sequence complementary to a 3' portion of the template. The primer comprises a plurality of deoxyribonucleotides and a ribonucleotide at its 3' terminal or 3' penultimate position.

The partially double-stranded/partially single-stranded structure is contacted with a mixture of deoxynucleoside α-triphosphate Sp isomers and a DNA polymerase for a time necessary

and under conditions conducive for the enzymatic synthesis of a PS-Rp oligodeoxynucleotide complementary to a 5' portion of the template, thereby forming a double-stranded product. In some embodiments, the deoxynucleoside α-triphosphate Sp isomers are selected from the group consisting of deoxyguanosine 5'-(α-thio) triphosphate, deoxyadenosine 5'-(α-thio) triphosphate, deoxycytidine 5'-(α-thio) triphosphate, and thymidine 5'-(α-thio) triphosphate. The term DNA polymerase" refers to any enzyme able to covalently link the 3' end of one deoxyribonucleoside to the 5' end of another deoxyribonucleoside, thereby synthesizing DNA. In specific embodiments, synthesis is accomplished with DNA polymerase I, T4 DNA polymerase, Taq DNA polymerase, or reverse transcriptase.

The double-stranded product is then cleaved after the ribonucleotide at the RNA/DNA junction such that the PS-Rp oligodeoxynucleotide is liberated from its primer and template. In some embodiments, cleavage occurs between the primer and the newly synthesized PS-Rp oligonucleotide when the ribonucleotide in the primer is at the 3' terminal position of the primer. In other embodiments, cleavage occurs between the 3' penultimate and 3' terminal nucleotide of the primer which has been extended as the PS-Rp oligonucleotide.

In some embodiments of the invention, cleavage is carried out with an endonucleolytic enzyme such as RNase A, RNase T1, RNase T2, RNase

U2, RNase N1, and RNase N2, while in other embodiments, cleavage is achieved with an alkaline. In specific embodiments, a strong alkaline or base such as a halide hydroxide selected from the group consisting of sodium hydroxide, ammonium hydroxide, lithium hydroxide, or potassium hydroxide, is used for this purpose.

Another aspect of the invention are stereospecific Rp oligonucleotides prepared according to the method of the invention.

Yet another aspect of the invention includes an oligonucleotide comprising a plurality of deoxyribonucleotides covalently linked with at least one stereospecific Rp phosphorothioate internucleotide linkage. Such stereospecific Rp phosphorothioate oligonucleotides are believed to be less mitogenic than Sp phosphorothioates or than a mixture of Rp and Sp phosphorothioates. In one embodiment, all of the deoxyribonucleotides are linked with stereospecific Rp phosphorothioate internucleotide linkages.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

FIG. 1A is a diagrammatic representation of one embodiment of the protocol for synthesizing a stereospecific PS oligonucleotide;

FIG. 1B is a diagrammatic representation of another embodiment of the protocol for synthesizing a stereospecific PS oligonucleotide;

FIG. 2A is an autoradiogram demonstrating the nuclease resistance of phosphodiester (PO), stereospecific phosphorothioate (PS-Rp), and synthetic phosphorothioate (PS) oligonucleotides in bovine serum;

FIG. 2B is an autoradiogram demonstrating the nuclease resistance of PO, PS-Rp, and PS oligonucleotides in human serum;

FIG. 3A is an autoradiogram demonstrating the nuclease resistance of PO, PS-Rp, and PS oligonucleotides in the presence of DNA polymerase I;

FIG. 3B is an autoradiogram demonstrating the nuclease resistance of PO, PS-Rp, and PS oligonucleotides in the presence of T4 DNA polymerase;

FIG. 4A is a graphic representation of the hybridization affinity of PS-Rp and PS oligonucleotides to complementary DNA and RNA targets, as measured by melting temperature (T_m);

FIG. 4B is a graphic representation of the hybridization affinity of PS-Rp and PS oligonucleotides to complementary DNA and RNA targets, as measured by circular dichroism;

FIG. 5 is a graphic representation of the ability of PS-Rp (◆) and PS oligonucleotides (□) to inhibit rHIV reverse transcriptase; and

FIG. 6 is an autoradiogram demonstrating the susceptibility of PS-Rp and PS oligonucleotides to RNase H digestion.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides for procedures for synthesizing stereospecific PS-oligonucleotides, and, in particular for preparing stereospecific (all Rp) oligonucleotide phosphorothioates using an enzymatic process. These Rp oligonucleotides appear to bind to proteins with less affinity and are believed to be less mitogenic than their non-stereospecific counterparts.

The procedure established for synthesizing stereospecific PS-oligonucleotides (all Rp) is outlined in FIGS. 1A and 1B. It has been shown that DNA polymerase uses only Sp diastereomers of 2'-deoxynucleotide 5'-(α-thio) triphosphate during extension by single phosphorothioate to produce Rp linkages (Eckstein (1985) *Ann. Rev. Biochem.* **54**: 367-402). However, such an extension was limited to incorporation of a single phosphorothioate linkage.

To carry out the synthesis of an entire stereospecific molecule, an appropriate template having the complementary nucleic acid sequence complementary to the desired sequence of the stereospecific molecule and a primer having a nucleic acid sequence complementary to a 3' portion of the template are designed. Synthesis of the template is carried out using any method known in the art to produce phosphodiester-linker oligonucleotides. One such useful method uses β-cyanoethyl phosphoramidite chemistry (Beaucage in

Protocols for Oligonucleotides and Analogs, Methods in Molecular Biology, Vol. 20 (Agrawal, ed.) Humana Press, Totowa, NJ (1993) pp. 33-61). For synthesis of the primer, a plurality of deoxyribonucleotide phosphoramidites and a 3'-ribonucleotide at the position shown in FIG. 1) are incorporated with the use of a ribonucleoside phosphoramidite as the "synthon" or building block. This synthon is introduced to enable cleavage of the synthesized stereospecific oligonucleotide from the primer. After synthesis, deprotection is carried out using an RNA protocol such as desilylation (Damha et al. in *Protocols for Oligonucleotides and Analogs*, Methods in Molecular Biology, Volume 20 (Agrawal, ed.) Humana Press, Totowa, NJ (1993) pp.81-114).

According to the method of the invention, the primer is annealed to the template by methods well known in the art, such as by heating for 3 minutes at 90°C, and then cooling for one hour at room temperature. The primer is then extended to form an oligonucleotide with a nucleic acid sequence complementary to the template. Extension of the primer is carried out with any enzyme capable of such synthesis, such as Taq DNA polymerase, DNA polymerase I (Pol I polymerase), T4 DNA polymerase, and reverse transcriptase using all four 2'-deoxynucleoside 5'-(α-thio) triphosphates, or analogs of 2'-deoxynucleoside 5'-(α-thio) triphosphates, as described by Eckstein (*Ann. Rev. Biochem.* (1985) **54**:367-402).

After extension, the newly synthesized stereospecific PS-Rp oligonucleotide is liberated by cleavage from the primer and release from the template. Liberation may be accomplished with any procedure that will cleave the PS-Rp oligonucleotide from its primer at the ribonucleotide and will cause the PS-Rp oligonucleotide to dissociate from its template. The ribonucleotide may be at the 3'-penultimate (FIG. 1A) or 3'-terminal position (FIG. 1B) of the primer before extension. Useful treatments include cleavage with a strong alkaline which digests RNA or with an endoribonuclease or other enzyme which cleaves at an RNA/DNA junction. Preferable alkalies include halide hydroxides such as sodium hydroxide, lithium hydroxide, ammonium hydroxide, and potassium hydroxide, with the most preferable being ammonium hydroxide or potassium hydroxide. Useful enzymes include RNase A, RNase T1, RNase T2, RNase U2, RNase N1, and RNase N2, which are commercially available.

After cleavage, the product is purified by chromatography, gel electrophoresis, ultracentrifugation, or any other method known in the art that separates single-stranded oligonucleotides from themselves and from double-stranded molecules. PAGE, for example, on a 20% polyacrylamide gel containing 8 M urea is useful for this purpose. The band containing the PS-Rp oligonucleotide is purified by locating it, for example, by UV shadowing, and excising and desalted it. Generally, if synthesis is carried out using about 8 nmoles of primer and 2.5 nmoles

of template, about 1 nmole of PS-Rp oligonucleotide product is obtained.

The oligonucleotides of the invention are useful as modulators of gene expression. For example, to determine the function of a particular gene, an oligonucleotide of the invention can be targeted to that gene in a tissue culture system and the effects of this modulation studied. If the object is to determine the role a particular protein plays in a certain biological process, the oligonucleotides of the invention can be used as a tool to suppress the production of the protein by targeting the nucleic acid that encodes the protein and then observing the biological effects brought about the suppression of the protein. In such a situation, it is not the integrated oligonucleotide that is being studied but the effects of suppressing production of the protein. Use of the oligonucleotides of the invention in this way provides an easily executed alternative to the laborious method of mutating the targeted gene, and thereby creating a deletion mutant.

In addition, the PS-Rp oligonucleotides of the invention, like their chiral Sp counterparts, are useful as stereochemical probes of phospho- and nucleotidyltranserase, as these oligonucleotides can distinguish between the incorporation of a deoxyribonucleotide into a growing polymer via a single bond cleavage at the phosphorus of the nucleotide triphosphate as the 3'-hydroxyl of the growing chain displaces the pyrophosphate, or via two bond cleavages if a

covalent nucleotidyl enzyme is involved (reviewed in Brody et al. (1981) *Biochem.* 20:1245-1252).

The comparable stability of a PS-Rp oligonucleotide prepared according to the method of the invention was determined by exposing it, as well as sequence-comparable PO- and PS-linked oligonucleotides, to a nuclease source such as human or bovine serum. The results in FIG. 2A show that PS-Rp oligonucleotides are still present after 6 hours and up to 24 hours incubation in bovine serum, whereas PO oligonucleotides are not. In human serum (FIG. 2B), PS-Rp and PS oligonucleotides are still present after 120 minutes of incubation, whereas PO-linked oligonucleotides were digested after 60 minutes of incubation.

Consistent results were obtained when PO, PS, and PS-Rp oligonucleotides were incubated with T4 DNA polymerase I (FIG. 3A) or with DNA polymerase (FIG. 3B). As shown in FIG. 3B, both PS and PS-Rp oligonucleotides were present after 4 hours of incubation in the presence of DNA polymerase I, while PO oligonucleotides were not present after 1 hour.

These results demonstrate that PS-Rp oligonucleotides are more stable than phosphodiester-linked oligonucleotides, and have stability comparable to PS oligonucleotides in bovine and human serum, and in the presence of DNA polymerase and T_4 DNA polymerase.

PS-Rp oligonucleotide and synthetic PS-oligonucleotide were also compared for their affinity for complementary DNA and RNA targets, as measured by melting temperature(T_m) and Circular dichroism. As shown in FIG. 4A, both PS-Rp and PS-oligonucleotides showed the same T_m with DNA target (51.8° and 51.9°). However, PS-Rp oligonucleotide had a higher T_m (68.9°) compared to synthetic PS-oligonucleotide (64°) with an RNA target, indicating that it has a greater binding affinity for its target. FIG. 4B demonstrates that the difference in CD spectra for RNA duplexes is more significant than that for DNA duplexes, indicating that RNA target is more sensitive to the conformational change of PS-Rp versus PS oligonucleotides at hybridization than the DNA target.

One known non-sequence-specific effect of PS oligonucleotides present as mixture of Rp and Sp diastereomers is to inhibit the enzymatic activity of reverse transcriptase. To determine whether PS-Rp isomers have the same inhibitory activity as the diasteromeric mixture, PS-Rp oligonucleotides and PS oligonucleotides were incubated individually with reverse transcriptase. As shown in FIG. 5, the PS-Rp oligonucleotide was found to be a stronger inhibitor of reverse transcriptase than the synthetic PS-oligonucleotide mixture.

The PS-Rp oligonucleotides were also subjected to RNase H digestion to determine if their ability to trigger this degradative response is comparable to that of the PS diastereomeric

mixtures. FIG. 6 shows that PS-Rp oligonucleotides are better substrates for RNase H than the synthetic PS-oligonucleotide mixture.

Furthermore, these oligonucleotides are able to hybridize to complementary nucleic acid sequences and taken up by cells. Thus, stereospecific PS-Rp oligonucleotides have similar if not improved antisense characteristics as a diastereomeric Rp + Sp mixture of PS oligonucleotides.

The following examples illustrate the preferred modes of making and practicing the present invention, but are not meant to limit the scope of the invention since alternative methods may be utilized to obtain similar results.

EXAMPLES

1. Synthesis of PS-Rp Oligonucleotides

In a 30 μl volume of 50 mM Tris, pH 9.0, 10 mM $MgCl_2$, 8 nmoles primer (d-GGTGGCTAGCGTAGrUC, (SEQ ID NO:2)) and 2.5 nmoles template (d-AGAAGGAGAGAGATGGGTGCGAGAGACTACGCTAGCCACC (SEQ ID NO:3)) were pre-annealed by heating at 90°C for 3 minutes and allowed to cool at room temperature for 1 hour. The reaction volume was increased to 1.5 ml containing 50 mM Tris, pH 9.0, 10 mM $MgCl_2$, 5 mM DTT, 100 mM deoxyguanosine 5'-(α-thio) triphosphate, 100 mM deoxyadenosine 5'-(α-thio) triphosphate, 200 mM deoxycytidine 5'-(α-thio) triphosphate and 200 mM thymidine 5'-(α-

thio) triphosphate (DuPont-NEN, Boston, MA). The reaction was initiated by the addition of 2500 units of sequence grade Taq DNA polymerase (Promega Corp., Madison, WI). The mixture was incubated for 4 hours at 37°C. The reaction mixture was desalted and treated with of ammonium hydroxide solution (28-30%) at 55°C for 24 hours, or with 0.3 M KOH at 37°C for 1 hour. In other tests, the desalted reaction mixture is alternatively treated with RNase A , RNase T1, RNase T2, RNase U2, RNase N1, or RNase N2, which are available from the Sigma Chemical Co. (St. Louis, MO) at 95°C for about 3 minutes. The product was dried and purified by PAGE on a 20% denaturing polyacrylamide gel. The product was excised from the gel under UV shadowing and eluted with 500 mM ammonium acetate and desalted by dialysis, as described in FIG. 1.

2. Nuclease Resistance of PS-Rp Oligonucleotides

The stability of phosphodiester-linked (PO), stereoregular (PS-Rp), and synthetic PS-oligonucleotide (PS) in bovine and human serum was tested as follows. 60 pmole of [^{32}P] 5'-end-labelled oligonucleotide was incubated in 100 ul of human serum (Sigma Chemical Co., St. Louis, MO) at 37°C. Aliquots of 20 μl were sampled at time 0, 30, 60, and 120 minutes, and then incubated with 20 μl of 20 mM Tris, pH 7.8, 10 mM NaCl, 10 mM EDTA, 0.5% SDS, and 100 μg proteinase K at 65°C for one hour. The samples were extracted by phenol/chloroform, ethanol precipitated, and then analyzed by PAGE in gels containing 20%

acrylamide, 8.3 M urea. The gel was fixed in a acetic acid/methanol/water (10:10:80), v/v/v) solution and dried for 3 hours before being subjected to autoradiography. The autoradiograms are shown in FIGS. 2A (bovine serum) and 2B (human serum).

Further comparative nuclease resistance studies were performed using T4 DNA polymerase, and DNA polymerase (Pol I). The DNA polymerase I assay was carried out in 20 μl volume containing 30 pmoles [^{32}P]-5′ end labelled PO, PS, or PS-Rp oligonucleotide, 50 nm Tris, pH 8.0, 5 mM $MgCl_2$, 5 mM DTT, 0.05% bovine serum albumin, and 5 units of DNA polymerase I (Worthington Biochemical Corp., Freehold, NJ). An aliquot was removed at time 0 and the remaining was incubated at 37°C. 5 μl aliquots of were removed at times 1, 2, and 4 hours. To each time point aliquot 6 μl stop solution (95% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added. The samples were then analyzed by PAGE using a 20% acrylamide, 8.3% urea gel. The results are shown in FIG. 3A.

The T4 DNA polymerase assays were carried out in 30 μl volume containing 45 pmoles [^{32}P]-5′ end labelled PO, PS, or PS-Rp oligonucleotide, 50 mM Tris, pH 8.0, 5 mM $MgCl_2$, 5 mM DTT, 0.05% bovine serum albumin, and 7.5 units of T4 DNA polymerase (Promega, Madison, WI). An aliquot was removed at time 0 and the remaining was incubated at 37°C. 5 μl aliquots were removed at times 5, 15, 30, and 60 minutes. To each time point aliquot were added

6 μl stop solution (95% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The samples were then analyzed by PAGE in a 20% acrylamide, 8.3% urea gel. The results are shown in FIG. 3B.

3. Melting Temperature Studies

Melting temperature (T_m) studies were carried as follows. 1.06×10^{-6} M PS-Rp or PS oligonucleotides were placed in 10 mM phosphate, 100 mM NaCl, pH 7, subjected to temperatures ranging from 30 to 75°C, and the absorbance at A^{260} measured using a spectrophotometer (GBC 920, GBC, Victoria, Australia). The results are shown in FIG. 4A.

4. Circular Dichroism Studies

Circular dichroism studies were performed to determine the relative binding affinity of PS-Rp and PS oligonucleotides to complementary RNA or DNA sequences. 1.06×10^{-6} M PS-Rp or PS oligonucleotides were placed in 10 mM phosphate, 10 mM NaCl, pH 7.0, and subjected to 200-320 nm UV light using a spectropolarimeter (J-710, Jasco, Easton, MD). The results are shown in FIG. 4B.

5. Inhibition of rHIV Reverse Transcriptase

In order to determine the relative ability of PS-Rp oligonucleotides to inhibit reverse transcriptase, the following assay was performed. A 10 μl reaction mixture containing 1 mM of the

preannealed [^{32}P]-5'-end labelled primer (d-GATTCAGCTAGTCCA (SEQ ID NO:5)) and template, (d-CCAACTGTGATACGATGGACTAGCTGAATC (SEQ ID NO:6)), 50 mM Tris, pH 8.3, 10 mM $MgCl_2$, 50 mM KCl, 5 mM DTT, 0.25 mM deoxynucleoside 5'-triphosphates (dNTPs) and various concentrations of oligonucleotide was prepared. After the addition of 0.8 units rHIV reverse transcriptase (Worthington Biochemical Corp., Freehold, NJ), the mixture was incubated at 37°C for 1 hour. The reaction was arrested with 10 ml formamide and then analyzed by a 20% denaturing polyacrylamide gel electrophoresis. CPMs were measured for the extension product and remaining primer bands, and the percentage of inhibition was calculated. The results are shown in FIG. 5.

6. RNase H Activity Assay

1 pmole of [^{32}P]-3'-end labelled 39-mer HIV-I gag RNA (AGAAGGAGAGAGAUGGGUGCGAGAGCGUCAGUAUUAAGC (SEQ ID NO:3)) was preannealed with 10 pmole of PS-Rp or PS oligonucleotide in 10 ml buffer (20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM DTT, 5% glycerol). The reaction was brought up to a volume of 30 ml with the same buffer, and 40 units RNase H (Boehringer Mannheim Corp., Indianapolis, IN). An aliquot of 7 ml was removed as time (zero). 0.5 units of RNase H was added to the remainder of the mixture which was incubated at 37°C for 15 min. 7 μl aliquots were taken and quenched with 10 μl formamide at 1, 5, and 15 minute time points. The mixture was analyzed by PAGE on a 20% denaturing polyacrylamide gel which

was autoradiographed. The results are shown in FIG. 6.

7. Activity Assay

Activity of the PS-Rp oligonucleotides is measured by assaying their ability to inhibit HIV-1 expression in H9, MOLT-3, or other cells. In this assay, 5 x 10^5 cells per ml are infected with 2.5-5 x 10^8 virus particles of HIV-1 strains HTLV-IIIB or HTLV-IIIC. Infection with 500-1000 virus particles per cell represents a multiplicity of infection (MOI) of 0.5-1. Infection of cells is carried out by simultaneous addition of virus and antisense oligomers to the cells cultured in medium containing RPMI 1640 (10% (vol/vol) fetal bovine serum, 2 mM glutamine, and 250 μl of gentamicin per ml) in a humidified atmosphere containing 5% CO_2 at 37°C. After 4 days, the cells and supernatant are examined for the level of HIV-1 expression by measuring syncytia (MOLT-3 cells) and viral antigen expression as well as cell viability. The number of syncytia formed in MOLT-3 cells is counted after triturating the cells to obtain an even distribution of the syncytia in the culture. The average number of syncytia is obtained by counting several fields in duplicate cultures. Cell viability is measured in the presence of trypan blue, and the cells that excluded the dye are counted as viable cells. HIV-1 antigen expression is measured in cells fixed in methanol/acetone. Briefly, the cells were pelleted and then resuspended in phosphate-buffered saline (PBS) at a concentration of 10^6

cells per ml. The cells are then spotted on toxoplasmosis slides, air-dried, and fixed in methanol/acetone (1:1, vol/vol) for 15 min at room temperature. The slides are next incubated with 10% normal goat serum at room temperature for 30 min and washed with PBS (four times). HIV-1 p24 or p17 monoclonal antibody are added to each well and the slides incubated for 30 min in a humid chamber at 37°C. The slides are washed with PBS (four times), incubated with fluorescein isothiocyanate-labeled goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) for 30 min at 37°C, and then washed with PBS overnight. The slides are counterstained with Evan's blue, washed with PBS, mounted with 50% glycerol, and examined with a Zeiss fluorescence microscope. The percentages of cells fluorescing in the oligomer-treated and untreated cultures are compared.

8. Cellular Uptake

Culture dishes of Human 293, HeLas$_3$, and NIH3T3 subconfluent cells plated 24 hr previously in Dulbecco-modified Eagle's medium (DMEM), or of MDCK or HEp-2 cells grown in DMEM/Earle's balanced salt solution media (5 x 10^5 cells/ml; 2 ml in total) are incubated with ^{35}S-labelled PS-Rp oligonucleotide for 16 hr. The fractionation of the cells is carried out as follows. Cells are removed from the culture plate with trypsin, harvested (1,500 rpm, 5 min, 4°C), and washed twice with 4 ml of cold phosphate-buffered saline (PBS). The cells are lysed for 10 min at 4°C in 450 μl of buffer A (10 mM Tris-HCl, pH 7.4, 150 mM

NaCl, 1.5 mM $MgCl_2$) and 50 µl of 5% Nonidet P-40 (NP-40). After centrifugation at 2000 rpm for 5 min, the upper cytoplasmic fraction (combined cytosol and cell membrane) is removed and the pellet (nuclei) dissolved in 500 µl of buffer A and measured for radioactivity.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: SYNTHESIS OF STEREOSPECIFIC OLIGONUCLEOTIDE PHOSPHOROTHIOATES

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(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kerner, Ann-Louise
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(C) REFERENCE/DOCKET NUMBER: HYZ-028PCT

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCTCGCACC CATCTCTCTC CTTCT 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA/RNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGTGGCTAGC GTAGUC 16

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGAAGGAGAG AGATGGGTGC GAGAGACTAC GCTAGCCACC 40

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGAAGGAGAG AGAUGGGUGC GAGAGCGUCA GUAUUAAGC 39

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GATTCAGCTA GTCCA 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCAACTGTGA TACGATGGAC TAGCTGAATC 30

What is claimed is:

1. A method of synthesizing an oligonucleotide with stereospecific Rp phosphorothioate (PS) internucleotide linkages comprising the steps of:

(a) annealing a primer to a template, thereby forming a partially double-stranded/partially single stranded structure,

the template having a 5′ terminus and a 3′ terminus and comprising a plurality of deoxyribonucleotides, and

the primer having a 3′ terminus and a 5′ terminus and comprising a plurality of deoxyribonucleotides and a ribonucleotide at the 5′ terminus or at a 5′ penultimate position, the primer having a nucleic acid sequence complementary to a 5′ portion of the template;

(b) contacting the structure with a mixture of deoxynucleoside α-triphosphate Sp diastereomers and a DNA polymerase for a time necessary and under conditions conducive for the enzymatic synthesis of a PS-Rp oligodeoxynucleotide extension complementary to a 5′ portion of the template, thereby forming a double-stranded product;

(c) cleaving the double-stranded product after the ribonucleotide such that the PS-Rp oligodeoxynucleotide is liberated from the template and the primer.

2. The method of claim 1 wherein the DNA polymerase is selected from the group consisting of T4 DNA polymerase, DNA polymerase I, Taq DNA polymerase, and reverse transcriptase.

3. The method of claim 1 wherein the PS-Rp oligodeoxynucleotide is liberated from the template and the primer by cleavage after the ribonucleotide with an alkaline or an endoribonuclease.

4. The method of claim 3 wherein the alkaline is a halide hydroxide salt selected from the group consisting of ammonium hydroxide, sodium hydroxide, lithium hydroxide, and potassium hydroxide.

5. The method of claim 3 wherein the base is an enzyme selected from the group consisting of RNase A, RNase T1, RNase T2, RNase U2, RNase N1, and RNase N2.

6. The method of claim 1 wherein the deoxynucleoside α-triphosphate Sp diastereomers are selected from the group consisting of deoxyguanosine 5′-(α-thio) triphosphate, deoxyadenosine 5′-(α-thio) triphosphate, deoxycytidine 5′-(α-thio) triphosphate, and thymidine 5′-(α-thio) triphosphate.

7. The method of claim 1 wherein the ribonucleotide in the primer is at the 3′ terminus.

8. The method of claim 1 wherein the ribonucleotide in the primer is at the 3' penultimate position.

9. A PS-Rp oligonucleotide prepared according to the method of claim 1.

10. An oligonucleotide comprising at least two stereospecific Rp phosphorothioates.

11. An oligonucleotide comprising a plurality of deoxyribonucleotides covalently linked with at least one stereospecific Rp phosphorothioate internucleotide linkage.

12. The oligonucleotide of claim 11 in which all of the deoxyribonucleotides are linked with stereospecific Rp phosphorothioate internucleotide linkages.

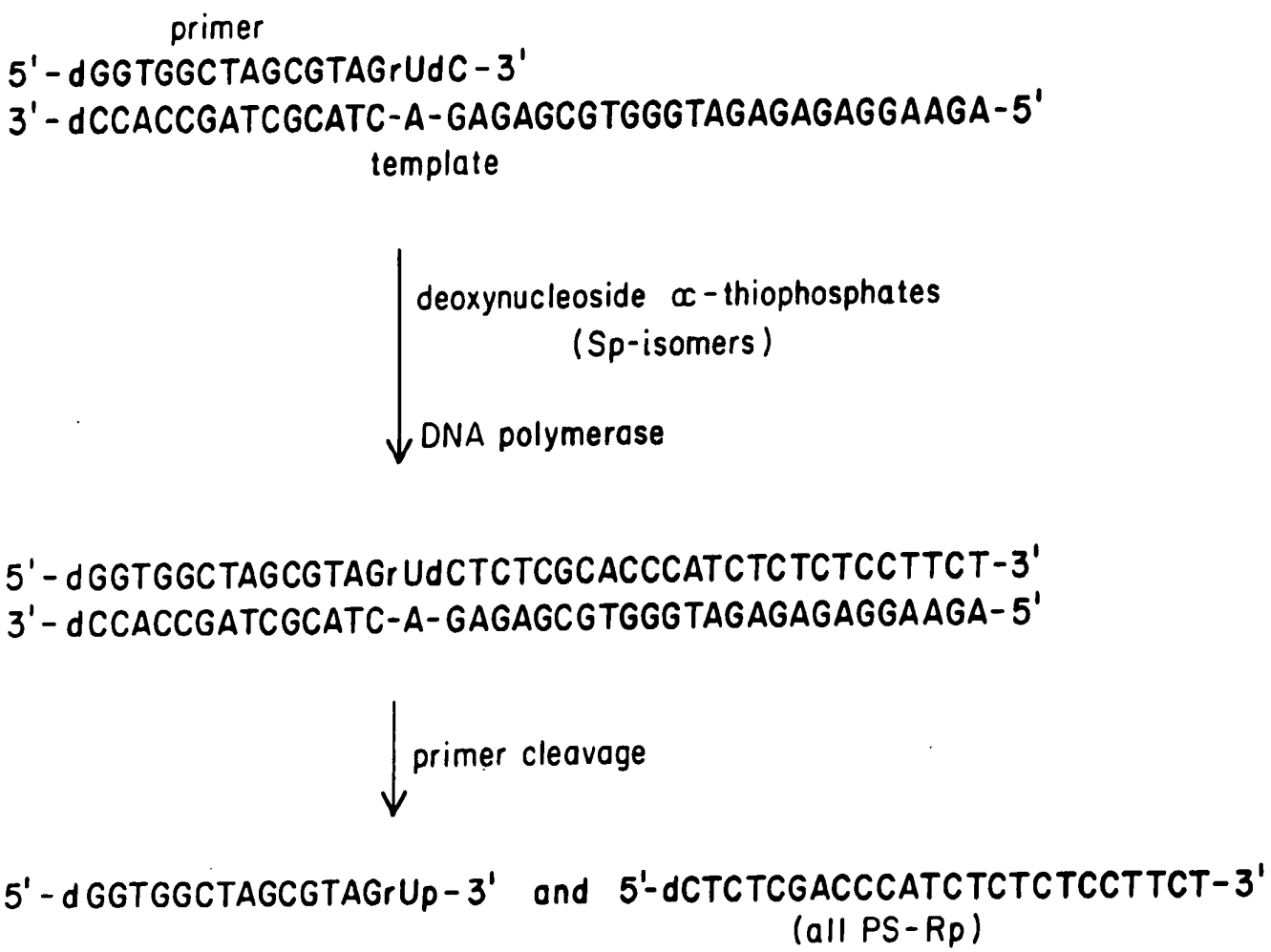
primer
5'-dGGTGGCTAGCGTAGrUdC-3'
3'-dCCACCGATCGCATC-A-GAGAGCGTGGGTAGAGAGAGGAAGA-5'
template
deoxynucleoside α-thiophosphates
(Sp-isomers)
DNA polymerase
5'-dGGTGGCTAGCGTAGrUdCTCTCGCACCCATCTCTCTCCTTCT-3'
3'-dCCACCGATCGCATC-A-GAGAGCGTGGGTAGAGAGAGGAAGA-5'
primer cleavage
5'-dGGTGGCTAGCGTAGrUp-3' and 5'-dCTCTCGACCCATCTCTCTCCTTCT-3'
(all PS-Rp)

FIG. 1A

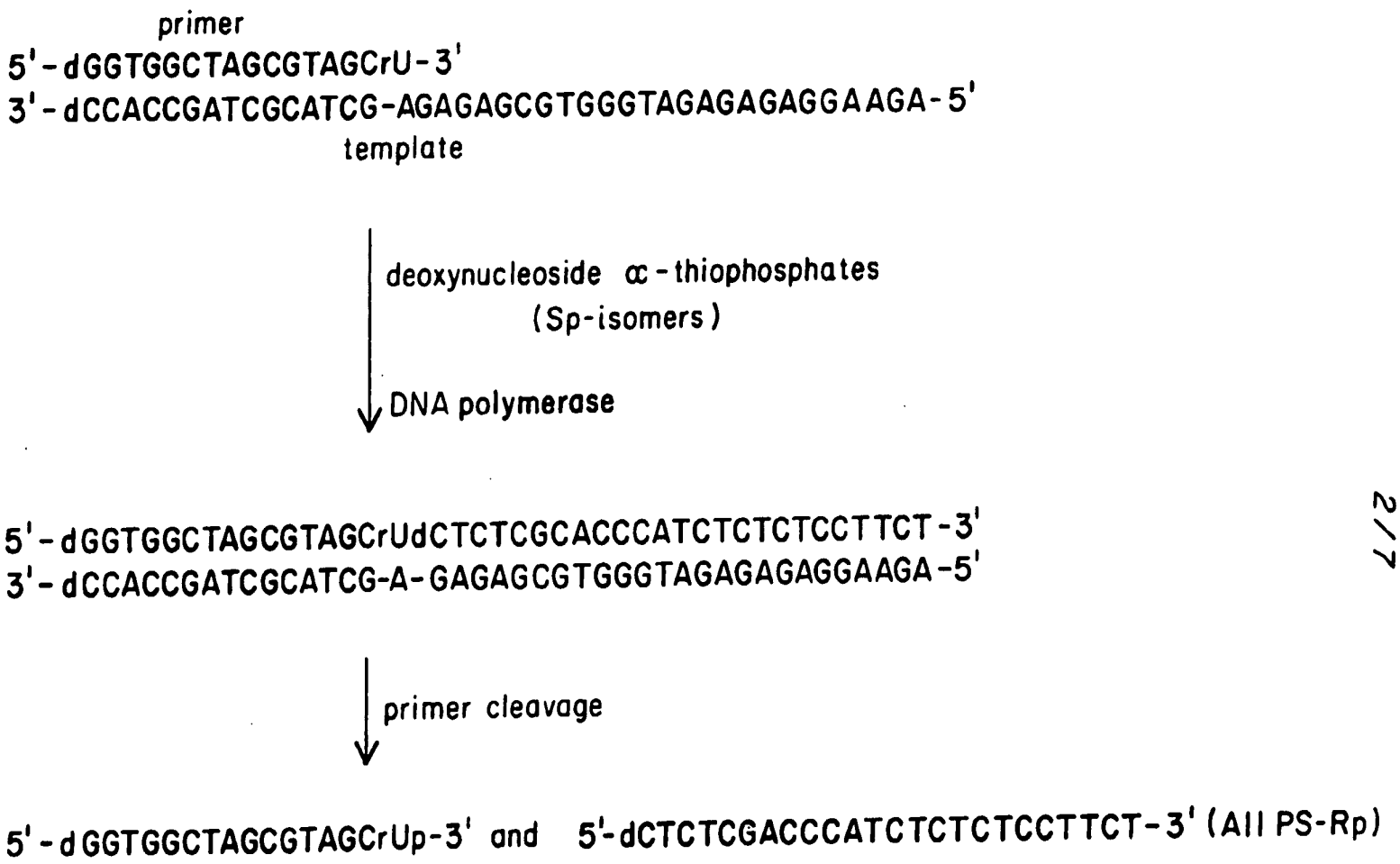
primer
5'-dGGTGGCTAGCGTAGCrU-3'
3'-dCCACCGATCGCATCG-AGAGAGCGTGGGTAGAGAGAGGAAGA-5'
template
deoxynucleoside α-thiophosphates
(Sp-isomers)
DNA polymerase
5'-dGGTGGCTAGCGTAGCrUdCTCTCGCACCCATCTCTCTCCTTCT-3'
3'-dCCACCGATCGCATCG-A-GAGAGCGTGGGTAGAGAGAGGAAGA-5'
primer cleavage
5'-dGGTGGCTAGCGTAGCrUp-3' and 5'-dCTCTCGACCCATCTCTCTCCTTCT-3' (All PS-Rp)

FIG. 1B

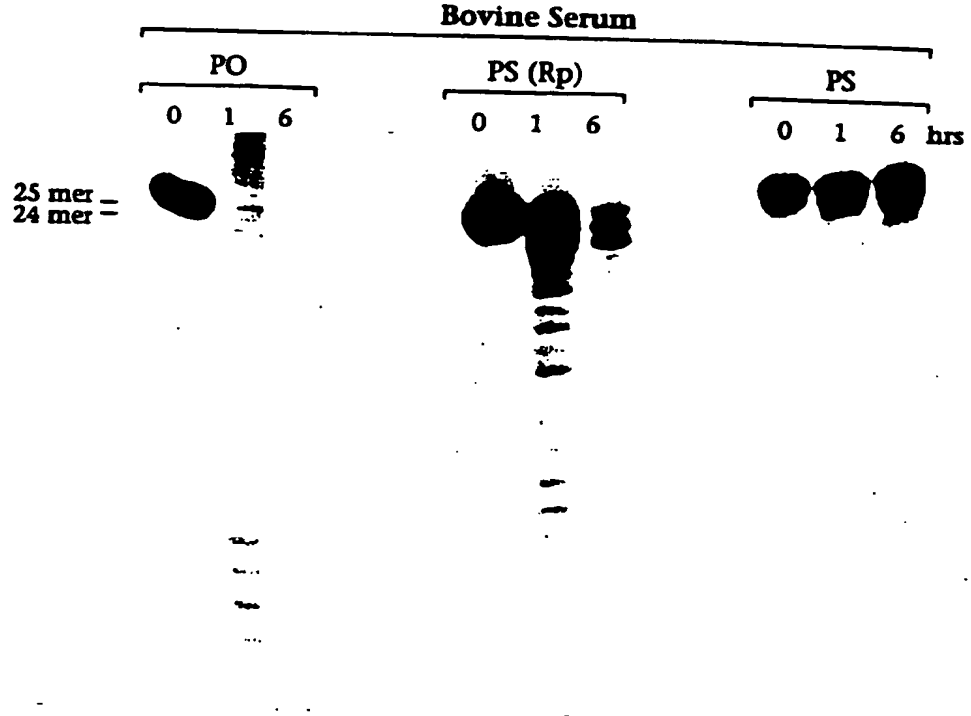
Bovine Serum
PO
PS (Rp)
PS
0 1 6
0 1 6
0 1 6 hrs
25 mer
24 mer

FIG. 2A

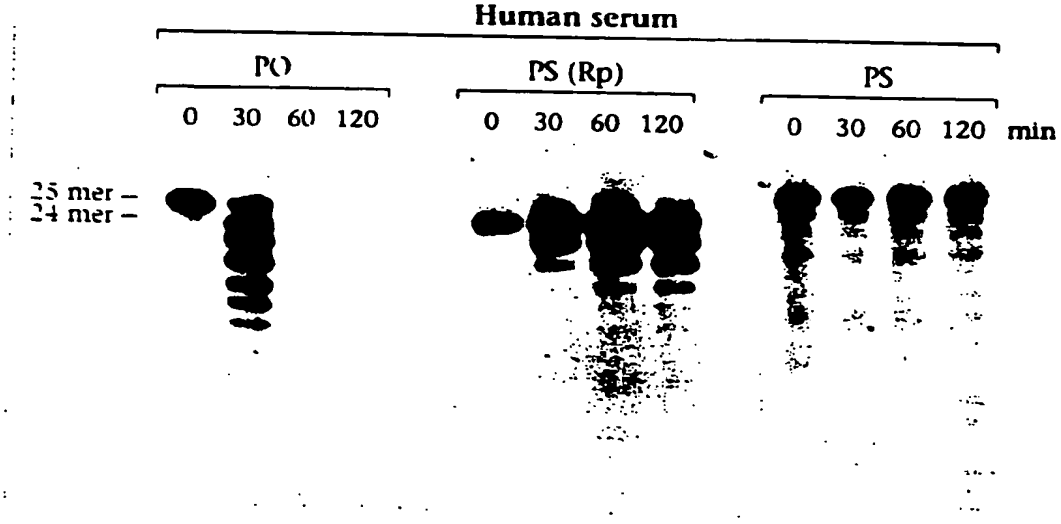
Human serum
PO
PS (Rp)
PS
0 30 60 120
0 30 60 120
0 30 60 120 min
25 mer
24 mer

FIG. 2B

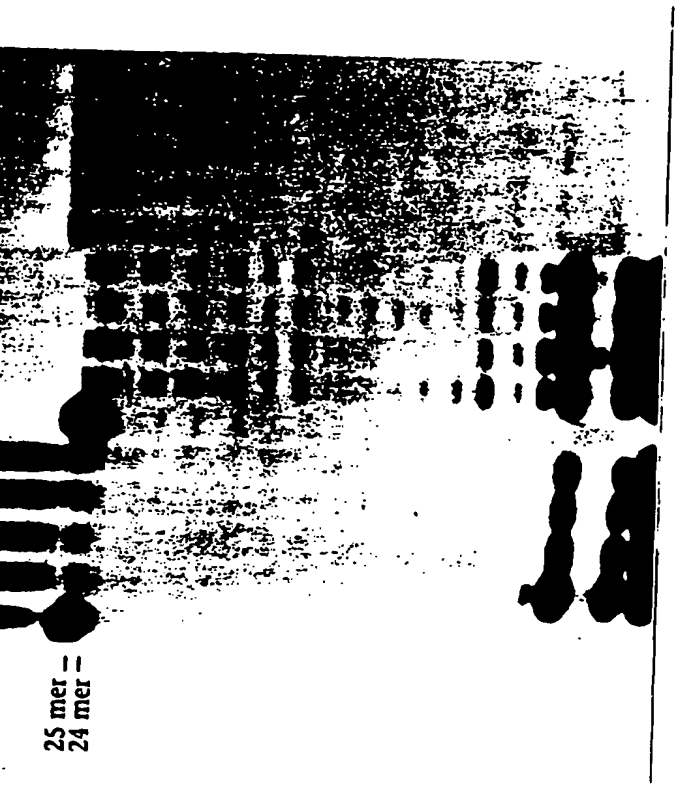
T4 DNA Polymerase
PO
0 5 15 30 60
PS (Rp)
0 5 15 30 60
PS
0 5 15 30 60 min
25 mer
24 mer

FIG. 3B

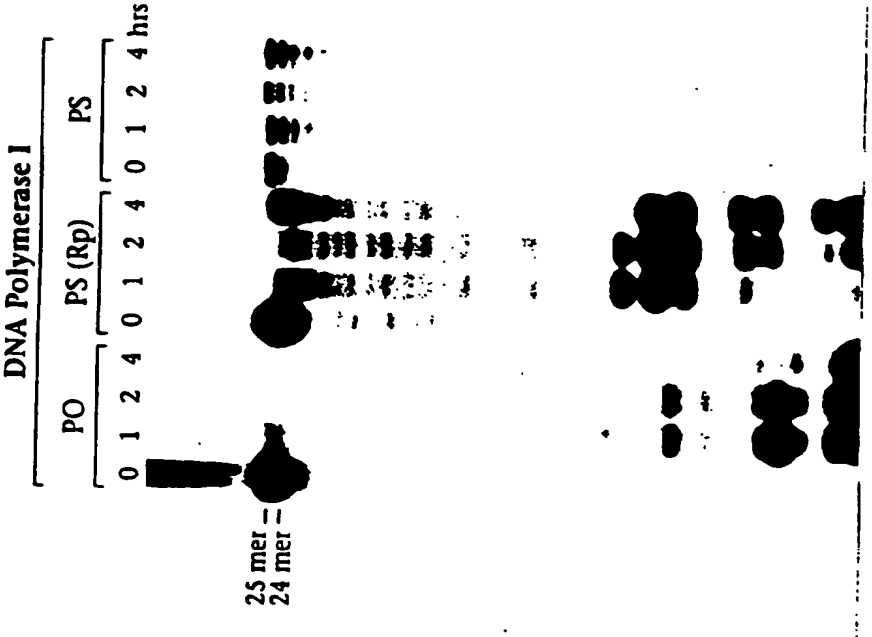
DNA Polymerase I
PO
0 1 2 4
PS (Rp)
0 1 2 4
PS
0 1 2 4 hrs
25 mer
24 mer

FIG. 3A

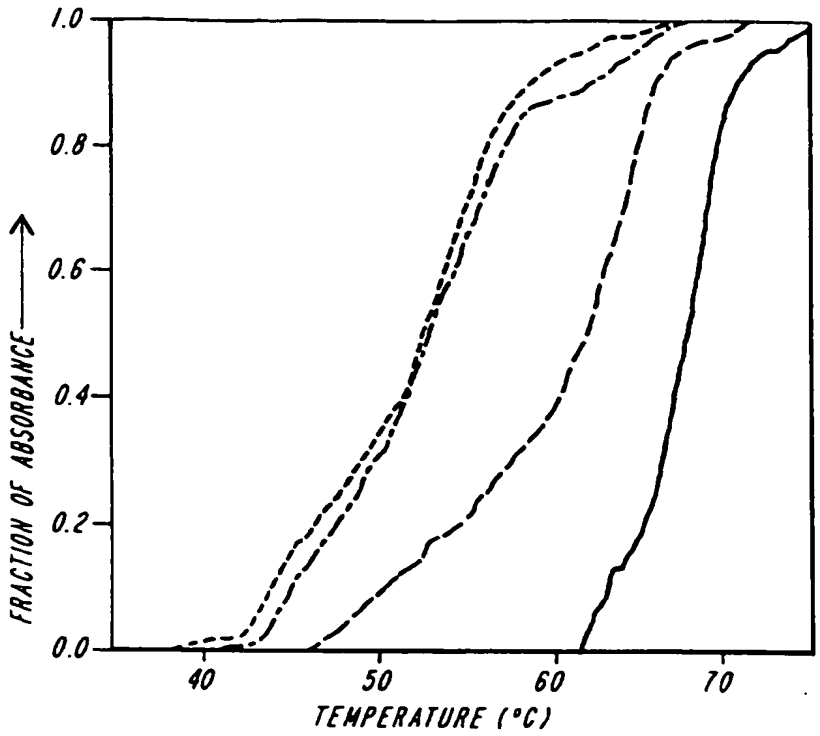
1.0
0.8
0.6
0.4
0.2
0.0
FRACTION OF ABSORBANCE
40
50
60
70
TEMPERATURE (°C)

FIG. 4A

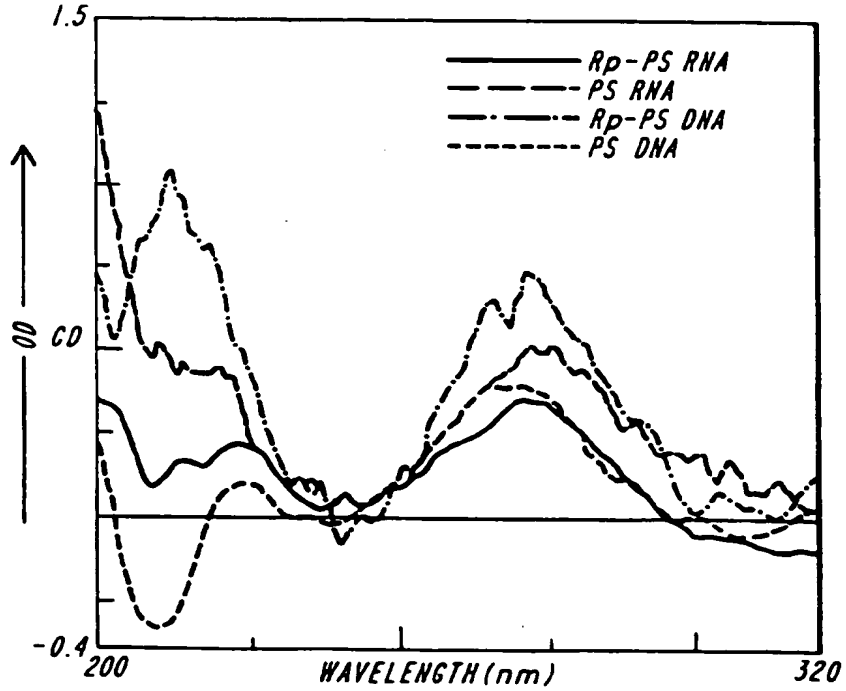
1.5
-0.4
OD CD
200
320
WAVELENGTH(nm)
Rp-PS RNA
PS RNA
Rp-PS DNA
PS DNA

FIG. 4B

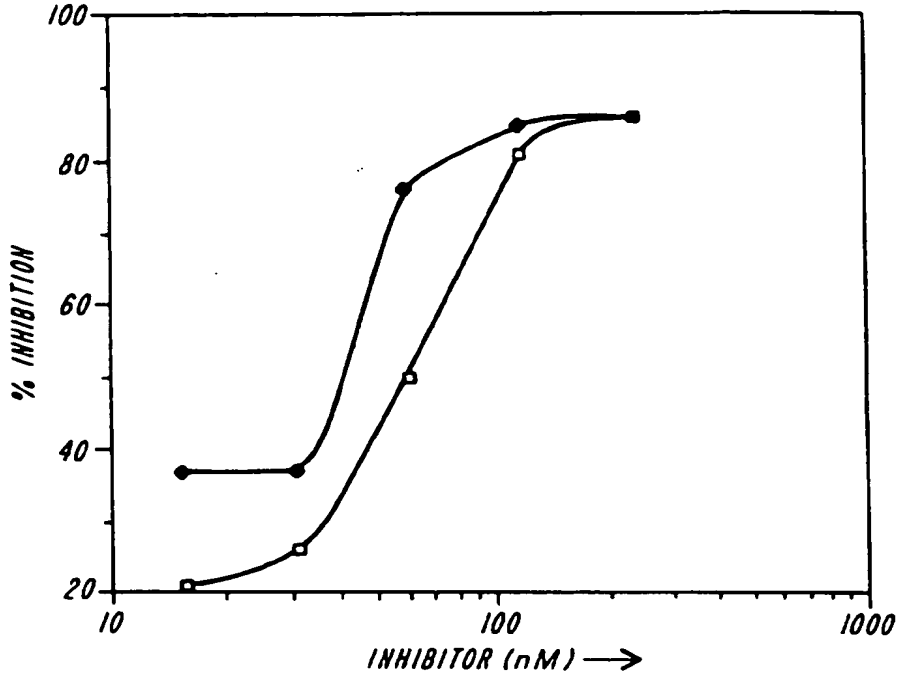

FIG. 5

FIG. 6